Protective effect of Thymoquinone and vitamin E on unilateral testicular torsion/detorsion in rats.

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Abstract

Aim: this work was designed to investigate the effects of Thymoquinone and vitamin E pretreatment after 2 h of unilateral testicular torsion and their possible mechanism of action.

Methods: the present study was performed on fifty male albino rats which were divided into five groups. Thymoquinone treated torsion/detorsion group, received pretreatment with Thymoquinone 100 mg/kg intraperitoneally 30 minutes before detorsion. Vitamin E treated torsion/detorsion group); received pretreatment with vit.E 100 mg/kg intraperitoneally 30 minutes before detorsion. Combined Thymoquinone and vit.E treated torsion/detorsion group received pretreatment in a dose of 100 mg/kg for each intraperitoneally 30 minutes before detorsion.

Results: thymoquinone and vitamin E pretreatment caused significant decrease of malondialdehyde and nitric oxide levels and significant increase in reduced glutathione and glutathione peroxidase levels compared with untreated torsion/detorsion group. They improved testicular structures as they caused significant decrease in congestion, edema, and damage of seminiferous tubules in the ipsilateral and contralateral testis histologically when compared with torsion/detorsion group. Conclusion: the results suggest that early administration of Thymoquinone and vitamin E might have a protective effect for preventing testicular injury caused by testicular torsion probably through their antioxidant effects.

Keywords

- Thymoquinone
- Vitamin E
- Testicular torsion/detorsion
- Antioxidants

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INTRODUCTION

It is well known that ischemia/reperfusion (I/R) generates metabolic and structural tissue damage, which may be due to trauma or sepsis. Reactive oxygen species (ROS) are over-produced on reperfusion, and play a critical role in the injury caused by ischemia-reperfusion (1). Oxidative stress is generated and contributes to reversible and irreversible cell injury (2).

Testicular torsion is a common emergency especially among children and adolescents. Testicular torsion and detorsion induces biochemical and morphological changes caused by IR injury in the testicular tissue (3).

Many mechanisms like in I/R injuries, ROS, and activation of inflammatory cytokines have been implicated in the testicular damage following torsion and detorsion process (4). I/R injuries are associated with the activation of neutrophils and inflammatory cytokines and injury starts in the ischemia period and exacerbates during reperfusion (5).

The rate of testicular injury is directly proportional to the duration of torsion, and early diagnosis followed by detorsion is the current management for the preservation of spermatogenesis and fertility (6). Although reperfusion is essential for the survival of an ischemic tissue, there is good evidence that reperfusion itself causes the pathophysiological cascades including an activation of neutrophils, inflammatory cytokines, and adhesion molecules with increased thrombogenicity, release of massive intracellular Ca2+, and ROS generation (7,8). ROS cause DNA damage, endothelial damage, and germinal cell necrosis.

Testicular torsion is the twisting of the spermatic cord, which cuts off the blood supply to the testicle and surrounding structures within the scrotum. In these circumstances, there is an increased risk of decreased sperm production, fertility, and atrophy of testis (9).

Experimental testicular torsion models showed that it induces apoptosis and damage in the ipsilateral and contralateral testes (10), although some authors suggested that the injury occurs only in the ipsilateral testis while the contralateral one is not affected (11).

Thymoquinone is one of the most important active principles of Nigella sativa seeds. Protection against reperfusion injury can be induced by assorted treatments including administration of antioxidants and anti-inflammatory drugs. Various therapeutic effects, such as antioxidant, anti-inflammatory, anticancer, antihistaminic, and antibacterial effects, have been described for Nigella sativa seeds (12,13). Additionally, it has been shown that Nigella sativa may have a possible protective effect against IR injury to various organs (14,15,16).

Vitamin E is the most prevalent nutritional antioxidant and has been shown to retard atherosclerosis in animal models (17). In addition to its antioxidant properties, vitamin E is anticancer and reduces the cytotoxic effect of oxidized lipoproteins, smooth muscle cell proliferation, platelet adherence and aggregation, inflammation, beside improving endothelial function (18). Vitamin E antioxidant activity has been suggested to scavenge ROS and attenuate IR injury (19).
MATERIALS AND METHODS

Animals
Fifty adult male albino rats weighing 200 - 250 g were provided by the Animal House of Zoology Department, Faculty of Science, Tanta University. The rats were housed in an animal room maintained at 23 ± 1 °C with a 12 h light/dark cycle, and they were allowed free access to water and food. The rats were acclimatized to housing conditions for at least 4 days before surgery.

Surgical procedure and drug administration
The rats were randomly divided into five groups. Group 1 (control group) with sham operation this group included (n= 10) sham rats. Under ether anesthesia, median scrotal incision was done, delivery of the right testis without twisting the testicle and then fixed within the scrotum.

Group 2 (n=10 rats) underwent 2 h of testicular torsion then detorsion; (untreated T/D group), torsion was created by rotating the testis 720° in a clockwise direction for 2 h. Then, the right testis was detorted and replaced into the scrotum.

Group 3 (T/D + Thymoquinone - TQ, Sigma/Aldrich, Deisenhofen, Germany, TQ group), torsion and detorsion as in group 2 but group 3 received pretreatment with Thymoquinone 30 minutes before detorsion in a single dose of 100 mg/kg by intraperitoneal injection.

Group 4 (T/D + vitamin E group);as in group 2 but group 4 received pretreatment with vit. E 30 minutes before detorsion in a single dose of 100 mg/kg by intraperitoneal injection.

Group 5 (T/D + Thymoquinone and vit. E treated group) torsion and detorsion as in group 2 but group 5 received pretreatment with Thymoquinone and vit. E before detorsion in a single dose of 100 mg/kg for each by intraperitoneal injection.

The animals were sacrificed and the blood was collected to evaluate serum testosterone hormone level by biochemical analysis. Malondialdehyde, nitric oxide, reduced glutathione and glutathione peroxidase levels were determined in testicular tissues as follow:

Biochemical analyses
Sample preparation
Testes tissues for the estimation of tissue antioxidant levels were prepared at 4°C. Tissues were weighted and cut into small pieces. 10% homogenate was made in ice-cold potassium phosphate buffer solution (PBS, pH 7.4) containing 5 mM ethylene-diamine-tetraacetic acid (EDTA) using a glass homogenizer. The homogenate was centrifuged at 20000g for 10 minutes at 4°C and the supernatant was obtained for measurement estimation of different antioxidants and lipid-peroxides. The total protein levels for homogenates were estimated by the Biuret method (20).

Testicular tissue GSH determination
Reduced GSH was determined by the method of Beutler et al. (1963) (21). The supernatants were mixed with 4 ml phosphate buffer and 0.01 M dinitro2, 2-dithiobenzoic acid (DTNB). After shaking, its absorbance was measured at 412 nm within 10 min of the addition of DTNB against blank. The quantity of GSH in tissue samples was calculated using the standard GSH and results were given in mg GSH/g tissue using a concurrently run standard curve.
Testicular glutathione peroxidase (GPx)

Glutathione peroxidase in testicular tissue was determined by the method of Paglia and Valentine (1967)(22).

Testicular tissue MDA determination

MDA levels in the samples were determined indirectly by a previously described method of Draper and Hadley (1990)(23). Briefly, 0.5 mL of sample was pipetted into a 10 mL centrifuge tube and 2.5 mL of trichloro-acetic acid (20%) and 1.0 mL of thiobarbituric acid (0.6%) solution was added. The tubes were heated for 30 min in a boiling water bath and the reaction mixture was then cooled in an ice-bath followed by the addition of 4.0 mL of n-butanol. The tubes were mixed with a vortex and centrifuged at 1500 g for 10 minutes. The absorbance of the organic layer was measured at 535 nm.

Testicular tissue NO determination

The production of NO was determined indirectly by measuring the nitrite levels based on the Griess reaction. Samples were initially deproteinized with 75 mmol/L ZnSO4. After clean up, an aliquot of the sample was treated with copperized cadmium in glycine buffer at pH 9.7 to reduce nitrate to nitrite. The concentration of nitrite in this aliquot thus represented the total nitrate plus nitrite. In the Griess reagent, a chromophore with a strong absorbance at 545 nm is formed by the reaction of nitrite with a mixture of naphthyl-ethylenediamine and sulfanilamide (24).

Serum Testosterone

Serum testosterone level was measured according to Bricaire et al. (1991) (25).

Statistical analysis

Statistical analyses were accomplished using the Statistical Product and Service Solutions (SPSS) computer program (version 13.0). All results were reported as means + SD. The comparison of the results from the various experimental groups and their corresponding controls was carried out using a one-way analysis of variance (ANOVA) followed by multiple comparison procedures (Tukey test).

RESULTS

Oxidative stress markers (MDA, GPx, GSH and NO):

Concerning lipid peroxidation and tissue malondialdehyde (MDA), reduced glutathione (GSH) and glutathione peroxidase (GPx) (Fig.1 A, B, & C), the untreated T/D rats in group 2 promoted significant increases in right and left testicular malondialdehyde (MDA) (P < 0.001) and significant decrease in right and left testicular in the levels of reduced glutathione and glutathione peroxidase (P < 0.001) compared to sham operated rats.

Histopathological examination

Bilateral orchiectomies were performed for histopathological examination in all groups. All testes were fixed in formalin and embedded in paraffin blocks. Tissue sections were stained with hematoxylin–eosin (H&E). The light microscope histological examination was done using 100 or 200 magnification power.
Concerning Thymoquinone administration to T/D rats in group 3 (Fig.1 A, B, & C), significant increase (P < 0.001) was observed in ipsilateral (right) and in contralateral (left) (P < 0.01) testicular glutathione peroxidase (GPx) and reduced (GSH) glutathione levels and significant decreases in MDA levels in right testis (P < 0.001) and left testis (P < 0.01) compared to untreated T/D rats.

Concerning Vitamin E administration to T/D rats in group 4 (Fig.1 A, B, & C), significant increase (P < 0.001) was observed in right and left testicular GPx and GSH levels and significant decreases in MDA level (P < 0.001) in ipsilateral testis and (P < 0.01) were observed in contralateral testis compared to untreated T/D rats.

Concerning Thymoquinone and vit. E pretreatment to T/D rats in group 5, significant increase (P < 0.001) was observed in right and left testicular GPx and GSH levels and significant decrease in...
right and left testicular MDA level (P < 0.001) compared to untreated T/D rats were observed in group 2 (Fig.1 A, B, & C).

Concerning tissue nitric oxide (NO) levels (Fig.1 D), the nitrite levels of both ipsilateral (right) and contralateral (left) testes in group 2 increased significantly compared with sham group (P < 0.001). In ipsilateral testis, TQ administration in group 3 significantly decreased (NO) production before detorsion procedure compared to T/D group (P < 0.001). Vit. E pretreatment in group 4 significantly decreased NO production compared to T/D group (P < 0.001). Combined TQ & vit. E pretreatment in group 5 significantly decreased NO production before detorsion procedure compared to T/D group 2 (P < 0.001).

In contralateral testis, TQ administration non-significantly decreased (NO) compared to T/D group (P < 0.05; Fig.1 D). Vit. E pretreatment significantly decreased NO production compared to T/D group (P < 0.05; Fig. 2). While TQ & Vit. E pretreatment in group 5 significantly decreased NO production compared to T/D group (P < 0.01; Fig. 1 D).

Concerning serum testosterone level (Fig. 2), a significant decrease (P < 0.001) in serum testosterone was observed in untreated T/D rats compared to sham operated rats. Upon pretreatment with TQ to T/D rats, serum testosterone showed significant increase (P < 0.01) compared to untreated T/D rats, although the level was still lower than sham operated rats. Pretreatment with vit. E to T/D rats, serum testosterone showed insignificant increase (P < 0.05) compared to untreated T/D rats. Pretreatment with combination of TQ & vit. E to T/D rats, serum testosterone showed significant increase (P < 0.001) compared to untreated T/D rats.

**Histological examination**

(Group 1) sham or control group showed normal seminiferous epithelium with Sertoli cells and spermatogenic cells. The regions between the seminiferous tubules are occupied by highly vascularized loose connective tissue and interstitial cells (Fig. 3).

(Group 2) torsion/detorsion group showed severe damage in seminiferous tubules with acidophilic exudate in their lumina and destruction of basal membrane of some tubules with absence of epithelial lining, severe inter-tubular edema, and severe congestion in the right or ipsilateral testis (Fig.4 A). The torsioned testis and the contralateral testis showed empty vacuolar spaces between Sertoli cells and germ cells in multilayered seminiferous epithelium. The contralateral testis (left testis) showed mild congestion and edema (Fig. 5 A).

(Group 3) Thymoquinone treated group showed marked improvement in testicular tissue. Ipsilateral testis showed mild vacuolization in seminiferous epithelium and germ cell and slight inter-tubular edema and congestion (Fig.4 B). The contralateral testis showed slight congestion and edema (Fig. 5 B).

(Group 4) Vit. E treated group, ipsilateral testis, showed moderate vacuolization and inter-tubular edema. Also, there were moderate damage of tubular lining of epithelial and spermatogenic cells with acidophilic exudate, moderate congestion and
inflammatory cellular infiltrates (Fig. 4 C). The contralateral testis showed decrease lining of spermatogenic cells and edema (Fig. 5 C).

(Group 5) Combined TQ & vit. E treated group, ipsilateral testis, showed damage of some spermatogenic cells but most tubules looks normal with sperms in their lumina (Fig. 4 D). Intraepithelial vacuolization and inter-tubular edema in the ipsilateral and contralateral testis were minimal (Fig. 4 D, 5 D).

Figure 2: Effect of torsion/detorsion, TQ, vit. E and combined TQ & vit. E on serum testosterone level in the studied groups. “a” shows significant difference with the sham group. “b” shows significant difference with the torsion/detorsion group. “ns” shows no significant difference (all at $p < 0.05$).

Figure 3: Normal testicular tissue stained with Haematoxiline & Eosine (magnification is 100x).
**Figure 4:** Effect of torsion/detorsion (A), TQ (B), vit. E (C) and combined TQ & vit. E (D) on ipsilateral testis in studied groups stained by Haemtoxiline & Eosine (magnification is 200x).

**Figure 5:** Effect of torsion/detorsion (A), TQ (B), vit. E (C) and combined TQ & vit. E (D) on contralateral testis in studied groups stained by Haemtoxiline & Eosine (magnification is 200x).
DISCUSSION

Results of the present study indicate that testicular T/D induces progressive biochemical and histological changes as a consequence of I/R injury. In our study, data revealed a significant increase in testicular lipid peroxidation products (TBARS and MDA), nitric oxide (NO) contents and histological damage of spermatogenic cells. The effect of oxidative stress of I/R in right testes are demonstrated by significant increases in right and left testicular MDA levels and significant decreases in right and left testicular glutathione peroxidase, as well as decreases in reduced glutathione levels compared to sham operated rats. These results are in agreement with the study of Ergur et al. (2008) who reported that I/R caused similar effects in torsioned testes. The significant increase in MDA levels in untreated T/D rats compared to both sham and treated rats indicated that I/R of the testes promotes severe cell membrane peroxidation (3). Also, oxidative stress induces per-oxidative damage to the plasma membrane of sperm, which causes loss of sperm function (6,26). The antioxidant effects of TQ and vit. E was previously supported by the findings that both protect against oxidative damage via inhibition of lipid peroxidation and restoration of reduced glutathione levels, and glutathione peroxidase activity in erythrocytes (15).

The effect of unilateral torsion on the contralateral testes was controversial. Turner (1987) reported that ipsilateral torsion does not result in contralateral testicular damage in rats (11). However, previous studies demonstrated that biochemical and histologic parameters of the contralateral testes did not reveal any statistical differences (27). The results of the present study are against these findings, because unilateral testicular torsion had an adverse effect on the contralateral testis, which has been previously explained as immunologic and oxidative mechanisms (1). Although still little is known about mechanisms on the effect of ischemia on the contralateral testes, our findings indicates that unilateral testicular IR produces contralateral damage, congestion and edema with significant decrease in reduced glutathione levels and glutathione peroxidase activity and significant increase in lipoperoxidation.

Testicular damage increased after detorsion procedure in both testes of untreated group explained by multiple factors that are known to induce testicular apoptosis during testicular torsion. Neutrophils and macrophages recruited during reperfusion have been implicated as mediators of parenchymal injury, and because of their ability to release a variety of toxic materials such as ROS and inflammatory cytokines, they are able to induce testicular cell apoptosis. Also, damaged spermatozoa can trigger further oxidative damage and cause expansion of damage and apoptosis (10). The increased levels of cytokines indicate tissue inflammation. Secretion of TNF-α acts as pro-inflammatory cytokines that induce and activate IL-6 and stress-related intracellular and extracellular pathways. In this regard, it was shown that these cytokines were produced from testicular cells as well as activated interstitial macrophages (5).
In our study we found that the testes of the I/R group had a high level of inter-tubular edema, congestion and inflammation which was correlated with high levels of nitrite. Also, in the same group nitrite levels in ipsilateral and contralateral testes were significantly higher than the sham operated group. The high nitrite levels may have been a result of activation of nitric oxide synthase and stimulation of pro-inflammatory cytokines, secretion of tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6).

The present study confirmed that TQ pretreatment caused significant decrease in nitrite level in ipsilateral testis, while Vit. E pretreatment significantly decreased nitrite levels in both ipsilateral and contralateral testes. Furthermore, administration of TQ and Vit. E in combination caused significant decrease in nitrite levels in ipsilateral and contralateral testes as well.

Histopathological examination of sections of ipsilateral right testes in untreated T/D rats showed loss of germinal epithelium, absence of the sperm which denotes absence of spermatogenesis. Also, presence of cellular infiltration, exudates, congested blood vessels are further proofs of the dysfunction of the right testes. Contralateral testes affection are also observed, one theory postulates decrease in blood supply of contralateral testis as a reflex to an afferent stimulus, other authors proposed autoimmune reaction, release of acrosomal enzymes from contralateral testis, and apoptosis (28,29,30). So the preservation of twisted testes by the detorsion procedure might cause further deterioration by I/R injury, indicating the importance of the removal of the damaged testis to decrease histo-pathological damage of the contralateral side, or using a therapy to decrease the injurious effect of I/R. Thymoquinone either alone or in combination with vit. E after testicular torsion and before detorsion improved the ipsilateral and contralateral testes as also was observed by the histopathological analyses.

In our study, concerning serum testosterone level significant decrease in serum testosterone was observed in untreated T/D rats compared to sham operated rats. Upon pretreatment with TQ to T/D rats, serum testosterone showed significant increase compared to untreated T/D rats, although the level was still lower than sham operated rats. After pretreatment with vit.E to T/D rats, serum testosterone showed insignificant increase compared to untreated T/D rats, while pretreatment with combination of TQ & vit. E to T/D rats, a significant increase in serum testosterone levels when compared to untreated T/D rats was found. In conclusion, the results suggest that Thymoquinone and vitamin E have a potential protective effect for preventing testicular injury caused by testicular torsion. So, Thymoquinone either alone or in combination with vit. E after testicular torsion and before detorsion have a protective role in the early period of the testicular biochemical changes associated with I/R injury. They probably have anti-inflammatory and antioxidant effects against I/R injuries of testicular tissues.

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